Microsatellite Typing of Avian Clinical and Environmental Isolates of *Aspergillus fumigatus*

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Genotyping *Aspergillus fumigatus* isolates

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Abstract

Aspergillosis is one of the most common causes of death in captive birds. Aspergillosis in birds is mainly caused by *Aspergillus fumigatus*, a ubiquitous and opportunistic saprophyte. Currently it is not known if there is a link between the environmental isolates and/or human isolates of *A. fumigatus* and those responsible for aspergillosis in birds. Microsatellite typing was used to analyze 65 clinical avian isolates and 23 environmental isolates of *Aspergillus fumigatus*. The 788 genotypes obtained were compared to a database containing genotypes of approximately 2500 strains isolated from human clinical samples and from the environment. There appeared to be no specific association between the observed genotypes and the origin of the isolates (environment, human or bird). Eight genotypes obtained from isolates of diseased birds were also found in human clinical samples. These results indicate that avian isolates of *Aspergillus fumigatus* may cause infection in humans.

Introduction

Fungal infections due to *Aspergillus* species are a major cause of morbidity and mortality among certain species of birds, captive as well as free-ranging, independent of age or status of the immune system (Tell 2005; Beernaert *et al*., 2008). *Aspergillus fumigatus*, a ubiquitous and saprophytic fungus, is the major etiological agent responsible for aspergillosis (Tell 2005).

To investigate the genetic and the epidemiological relationship between environmental and clinical isolates, fingerprinting methods with high discriminatory power must be applied. Also interlaboratory reproducibility and objective interpretation of the fingerprinting data are highly recommendable (de Valk *et al*., 2007). All of these characteristics can be found in typing methods based on short tandem repeats (STRs), such as microsatellite length polymorphism (MLP) and STR*Af* typing (Bart-Delabesse *et al*., 1998; de Valk *et al*., 2005; de Valk *et al*., 2007; Vanhee *et al*., 2008a). While the discriminatory power is high in pattern-based techniques, such as random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, and amplified fragment length polymorphism (AFLP), STR*Af* typing proved to be more simple and reproducible (Bart-Delabesse *et al*., 1998; de Valk *et al*., 2005; de Valk *et al*., 2007; Vanhee *et al*., 2008a,b).

Previous molecular typing studies showed that there was a high variability among avian isolates and multiple genotypes were recovered from healthy and diseased birds (Lair-Fulleringer *et al*., 2003; Olias *et al*., 2009; Alvarez-Perez *et al*., 2010). However, currently it is not known if there is a link between environmental isolates and/or human isolates of *A. fumigatus* and those responsible for aspergillosis in birds.

Therefore, in this study STR*Af* typing was performed on environmental and avian clinical isolates of *A. fumigatus* and the results were compared with a database containing genotypes from *A. fumigatus* isolated from clinical human samples and from the environment.

Material and Methods

Isolates. Sixty-five clinical avian and 23 environmental isolates of *A. fumigatus* were subjected to STR*Af* typing. The clinical isolates were collected at 6 different institutes, 4 located in Belgium and 2 in The Netherlands and were obtained from birds, belonging to 13 orders, 18 families and 35 species (Table 1), that died from an *A. fumigatus* infection. To collect the environmental isolates, Sabouraud dextrose agar plates were placed at 20 different locations in the vicinity of Ghent, Belgium. After incubation for three days at room temperature, the plates were placed in an incubator at 37°C. Fast-growing greenish colonies were purified on Sabouraud dextrose agar.
The isolates were identified based on the macro- and micro-morphology of the fungus. Determination of partial DNA sequences of the beta-tubulin and rodletA genes (Alcazar-Fuoli *et al*., 2008) and the ability to grow at 48°C were used to confirm species identity.

STR*Af* assay. Fungal DNA was prepared from all isolates as described by Beernaert *et al*. (2008). PCR primers for the STR*Af*2, -3 and -4 panels and amplification conditions were as described by de Valk *et al*. (2005), except that FAM labelling was replaced by VIC labelling, HEX labelling by NED labelling and TET labelling by PET labelling. Allelic ladders were used with the same fluorescent labels as above for each of the three trinucleotide markers in the STR*Af*3 panel as described by de Valk *et*

al. (2009). The obtained PCR products were diluted 10-fold with distilled water. Two microliter of the diluted PCR products were added to 12 µl of formamide (Amresco Inc, Ohio, USA) and 1 µl of GS 500 LIZ size standard (Applied Biosystems, Halle, Belgium). Following denaturation of the samples for 2 min at 95°C and rapid cooling to 0°C for 30 min, they were injected onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Halle, Belgium) equipped with a 16 capillary array. Genemapper v3.5 (Applied Biosystems, Halle, Belgium) was used to determine the size of each amplified fragment. All results are reported as repeat numbers. Repeat numbers for the markers in the STR*Af*3 panel were determined by comparison to the allelic ladders. The repeat numbers of the markers in the STR*Af*2 and -4 panels were determined with the reference size values taken from the original publication and validated using a set of reference isolates with known genotypes (de Valk *et al.*, 2005).

Data analysis. Typing data was imported into BioNumerics version 6 software (Applied Maths, St-Martens-Latem, Belgium) and analyzed using the categorical multistate similarity coefficient with UPGMA clustering. The obtained genotypes were then compared to a database containing genotypes from approximately 2500 human and environmental *A. fumigatus* isolates from Europe.

Results

A total of 88 *A. fumigatus* isolates were analyzed using STR*Af* typing and a total of 78~~9~~ genotypes were obtained. Seventy genotypes were found once, 6 genotypes were found twice and 2 genotypes were found three times. The Simpsons index of diversity (D) for the clinical avian isolates was calculated to be 0.995.

In the dendrogram (Figure 1), 8 clusters can be identified, in which the genotype of the isolates only differed at a single locus. In one cluster, identical genotypes were found for a clinical avian isolate and an environmental isolate. Two clusters contained clinical and environmental isolates, four clusters only clinical isolates and two clusters only environmental isolates.

Comparing the results of our analysis to a database containing genotypes from approximately 2500 human and environmental isolates revealed that 59 genotypes in our collection of avian and environmental isolates were unique. Eight genotypes (clinical as well as environmental) belonging to 4 of the 8 clusters mentioned above were also present in the collection of 2500 isolates. Eight genotypes from clinical avian isolates were found in human isolates from the Netherlands, Switzerland and Germany and 9 genotypes were also found in environmental isolates from the Netherlands and Spain. Eight environmental genotypes from Belgium were identical to genotypes found in human or environmental isolates from the Netherlands, Spain, Germany, the United States of America and Norway.

Discussion

Previous epidemiological studies have investigated the origin of avian aspergillosis in limited geographical areas and in one species of birds (Lair-Fulleringer *et al*., 2003; Olias *et al*., 2009; Alvarez-Perez *et al*., 2010). This is the first study determining the genetic diversity among *A. fumigatus* isolates obtained from 35 avian species collected at 6 different institutes and environmental isolates collected at 20 different locations in Belgium.

The results of this study demonstrate that the genetic diversity among avian clinical isolates is extremely high. The 65 isolates, collected from 65 birds affected with

aspergillosis, belonged to 57 distinct genotypes. In contrast, Alvarez-Perez *et al*. (2010) reported 13 distinct genotypes in 33 isolates from 5 diseased birds and Lair-Fullinger *et al*. (2003) 23 distinct genotypes in 114 isolates from 30 healthy and 2 diseased turkeys. The low number of distinct genotypes in relation to the number of isolates in those studies could be explained firstly by the fact that a limited number of animals with aspergillosis were used. Secondly, the animals belonged to closed collections and multiple isolates from each animal were examined. Finally, the genotyping was performed with a method described by Bart-Delabesse *et al*. (1998) including only four microsatellite markers being less discriminatory than the panel of 9 microsatellite markers described here. Alternatively, these animals may have been exposed to a common source of material(s) that may have been contaminated with a limited diversity of *Aspergillus fumigatus* spores such as bird feed.

The environmental and avian isolates are widespread throughout the dendrogram, suggesting that any environmental isolate of *A. fumigatus* is possibly infectious to birds. This finding is supported by the study of Peden and Rhoades (1992), who inoculated isolates from diverse origins (environmental, mammalian, and avian) in air sacs of turkeys. All isolates were able to induce aspergillosis in these birds.

High reproducibility of the STR*Af* assay and the ease of interlaboratory exchange of the results allowed comparing the dataset of 108 clinical avian and environmental isolates to a dataset of 2500 genotypes from human and environmental isolates (~~Pasqualotto~~ de Valk *et al*., 200~~7~~9). There appeared to be no specific association between the observed genotypes and the origin of the isolates (environment, human or bird). This was expected because of the high degree of genetic diversity among *A. fumigatus* isolates, independent of the species or geographical region (Debeaupuis *et al*., 1997; Chazalet *et al*., 1998; Rosehart *et al*., 2002; Menotti *et al*., 2005; Klaassen

et al., 2009). Moreover, 8 genotypes derived from diseased birds were also isolated from humans with aspergillosis indicating that avian isolates of *A. fumigatus* could be considered infectious to humans.

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Figure legends

Figure 1: Dendrogram generated from genotyping 65 clinical and 23 environmental *A. fumigatus* isolates. Isolates recovered from clinical samples are denoted as K and strains isolated from the environment are denoted as O. Framed are the genotypes that show microvariation. The scale bars above the dendrogram indicates the percentage identity between the genotypes.

299 Table 1: Order, Family and Species of domestic and wild birds from which *A.*

300 *fumigatus* isolates were obtained.

Order	Family	Species	N° of isolates	Designation of isolates
Accipitriformes	Accipitridae	*Geranoaetes melanoleucus*	2	K30; K57
		Accipiter gentilis	2	K51; K58
Anseriformes	Anatidae	*Netta rufina*	2	K66; K76
		Melanitta nigra	2	K61; K82
		Mergus serrator	1	K67
		Anas platyrhynchos	1	K43
Charadriiformes	Alcidae	*Uria aalge*	2	K10; K11
		Fratercula artica	1	K64
	Laridae	*Larus marinus*	1	K37
Ciconiiformes	Ciconidae	*Ciconia ciconia*	1	K20
Columbiformes	Columbidae	*Columba livia*	6	K12;K24; K25; K36; K42; K73
Falconiformes	Falconidae	*Falco rusticolus*	4	K8; K22; K34; K52
		Falco peregrinus	1	K54
Galliformes	Phasianidae	*Gallus gallus domesticus*	4	K6; K15; K16;K72
		Polyplectron emphanum	1	K81
		Pavo cristatus	1	K4
		Rollulus rouloul	1	K65
Gruiformes	Gruidae	*Anthropoides paradiseus*	1	K75
Passeriformes	Corvidae	*Pica pica*	1	K21
		Corvus monedula	1	K23
	Cracticidae	*Gymnorhina tibicen*	1	K18
	Fringillidae	*Pyrrhula pyrrhula*	1	K45
	Sturnidae	*Leucopsar rothschildi*	1	K19
Piciformes	Ramphastidae	*Rhamphastos toco*	1	K46
Psittaciformes	Cacatuidae	*Cacatua sp*	1	K9
	Psittacidae	*Psittacus erythacus*	12	K7; K48; K55; K59; K60; K62; K68; K69; K70; K78; K79; K80
		Cyanoramphus spp	3	K3; K5; K49
		Amazona barbadensis	1	K63
		Pionus sp	1	K50
		Poicephalus senegalus	1	K56
		Neophema sp	1	K47

		Enicognathus leptorhynchus	1	K33
		Lathamus discolor	1	K39
Sphenisciformes	Spheniscidae	*Spheniscus sp*	1	K32
Strigiformes	Strigidae	*Athene cunicularia*	2	K17; K31

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